A Method for the Assay of Penicillin

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In an earlier publication (Abraham, Chain, Fletcher, Florey, Gardner, Heatley & Jennings, 1941) a very brief account of a method of assay of penicillin was given. A fuller account of this same method (with some minor modifications) is now presented for the following reasons. Experience has shown that the original instructions were not sufficiently detailed to enable other workers to use the method without difficulty. The method has been in regular use in this laboratory since 1940 and is still believed to be as satisfactory, for general use, as other methods. Slight modifications have increased its accuracy. Whatever progress is made in the chemical investigation of penicillin, a biological method of assay is likely to be necessary for some purposes for a considerable time because of its great sensitivity and specificity. This assay method has already been used in the investigation of other antibiotics, and it may have a wide application in this field.

The following account is confined strictly to the procedure used in this laboratory. Modifications have been proposed—sometimes by two or more groups of workers independently—and though some of these are mentioned below the author has had insufficient experience of them to justify a critical appraisal.

PRINCIPLE OF THE METHOD

An agar plate is seeded by pouring on a broth culture of the test organisms, draining and drying. Short open-ended cylinders of glass or vitreous porcelain are then placed on the surface of the agar and the solutions to be assayed are placed in the cylinders. After incubation the surface of the agar becomes covered with a confluent bacterial growth, except for a circular zone around each cylinder where the penicillin has diffused out and inhibited growth. The diameter of this zone of inhibition is related to the concentration of penicillin in the solution in the cylinder, and by setting up a standard containing a known concentration of penicillin at the same time as the unknown, the strength of the latter can be calculated.

PRACTICAL DETAILS OF THE METHOD

Pouring the plates. The Petri dishes (which should be of a uniform size and not too shallow) are poured to a depth of 3–5 mm. with a simple medium such as the following:

Lemco Extract of Meat	1%
Evans's Bacteriological Peptone	1%
Sodium chloride	0·5%
Agar	2%
Made up with tap water	

For the last 2 years it has been customary to add to this 50 ml./l. of M/1·5 phosphate buffer pH 6·8, and phenol red to a final concentration of 0·0025%. The presence of the buffer seems to give sharper zones of inhibition, but opinion is divided as to whether the inclusion of the indicator is an improvement. The medium is sterilized at 15 lb./sq. in. pressure for 20 min. in bottles containing 400 ml., the actual pouring of the plates being simplified and standardized by means of an automatic measuring head. The latter, which is sterilized separately, can be used with at least two successive bottles if ordinary precautions against contamination are taken. 20 ml. is a convenient amount of agar for the ordinary 4 in. Petri dish. Several dozen plates can be poured at one time and stored at room temperature for some days before use. Little infection is encountered nowadays, but at one time plates were all incubated for 24 hr. after pouring, those developing contaminants being discarded.

Seeding the plates. Staphylococcus aureus, no. 6571 of the National Collection of Type Cultures, is used as test organism, though presumably any penicillin-sensitive strain would serve. The plates are seeded by placing on each a small volume of a 16–24 hr. broth culture (or of a 10–100-fold dilution of such a culture). After being tilted and shaken so that the whole surface of the agar is completely covered with the liquid, the plates are left tilted at about 20° to the horizontal for a few moments and the surplus bacterial suspension is drawn off. This edge of the plate is marked (for reasons given below). The surface of the agar is then dried by setting the plates in the 37° incubator for 1–2 hr. with the lid lifted about ½ in. above the bottom of the dish.

Two types of drying-stand have been used; in one the lids rest on two glass rods supported at the correct height, and in the second type a wooden baseboard is fitted with sets of three inward-sloping springy wires which grip the lids and allow them to be set to any desired height over the bottoms of the dishes. The actual time required for

drying the plates depends on conditions of humidity, etc., in the incubator. Usually 1 hr. is sufficient, but in one incubator, although the surface of the agar was quite dry after this period, when the cylinders had been put on and the plate incubated again overnight, a ring of free moisture was found round each cylinder. This interfered with the interpretation of small zones of inhibition. The trouble disappeared when the plates were dried for 2 hr. instead of 1 hr.

The dried seeded plates can be kept in the ice-chest for at least 3 days.

Nearly 2 years ago attempts were made to apply the well-known principle of seeding the agar in bulk prior to pouring the plates, but at the time this modification was thought to offer no advantages and was discontinued. However, Foster & Woodruff (1943*a*) obtain excellent results with a bulk seeding technique, and have described (1943*b*) a further modification in which the agar is pre-seeded with a pasteurized suspension of spores of *B. subtilis*. Independent workers have confirmed that the zones of inhibition are exceptionally sharp, and there is less day-to-day variation in the standard curve, which is somewhat flatter than when *Staph. aureus* is used. Although the accuracy is thereby reduced, the effective range of the method is correspondingly increased.

Preparation of solutions for assay. A considerable practical advantage of the present method is that the solutions to be assayed need not be sterile, since contaminating bacteria are confined to the inside of the cylinder. It is readily conceivable, however, that heavy contamination by bacteria which actively destroy penicillin may reduce the concentration of the latter during the incubation and give falsely low or even negative values. For the same reason it is important that the pH of the solution should be within the range 5·0–8·0. The concentration of penicillin should be preferably between 0·5 and 2·0 units/ml. With other organisms, e.g. *B. subtilis*, a different range may be covered. M/50 phosphate buffer, pH 7·0, is used instead of water in preparing solutions, since traces of acid or alkali in the glassware might otherwise bring the pH to a dangerous level. Very strongly buffered solutions are to be avoided, but the presence of ether or chloroform seems to have no disturbing effect.

Placing and filling cylinders. The cylinders, having been dry-sterilized in a Petri dish, are picked up in forceps, momentarily flamed, and then carefully placed on the surface of the agar. There may be a very brief sizzling sound (though the cylinder should scarcely be hot enough to cause this) and a perfectly fluid-tight seal is made between the agar and the cylinder. The cylinders are *not* pressed into the agar. The solutions to be assayed are then placed in the cylinders, care being taken that there is no air space between the fluid and the surface of the agar. The exact volume of fluid seems to make little difference to the assay value, but in practice the fluid is filled level with the top of the cylinder.

After use the cylinders are placed in strong sulphuric acid containing a few crystals of sodium nitrate, and warmed, which both sterilizes and cleans them. They are well washed under the tap, then in distilled water and finally dry-sterilized in a Petri dish.

Specifications for cylinders. The cylinders may be made of glass or *vitreous* porcelain, glazed or unglazed. Provided they are of uniform size the actual dimensions are unimportant. (The cylinders we have used hitherto are 9·6 ±0·2 mm. high, by 5·1 ±0·2 mm. internal diameter and 7·2 ±0·1 mm. external diameter. One end is bevelled internally and the sharp edge is ground perfectly plane; the other end is coloured for easy identification. Cylinders of vitreous porcelain of this type are obtainable from James Macintyre and Co., Ltd., Burslem, Staffs.) Foster & Woodruff (1943*a*) have stated that the bevel is unnecessary. This is readily understandable as the bevel was introduced originally to give a better seal between the surface of the agar and the cylinder, which was at that time placed on the agar without warming. If the momentary flaming of the cylinders immediately before placing on the agar is adopted, the need for the absolutely plane edge and the desirability of the bevel both disappear.

Dr R. D. Coghill has suggested (private communication) that the medium be made up to contain less than 2% agar. When the cylinders are placed on the softer agar a good seal is obtained without preliminary flaming, thus saving both time and agar. Under these conditions the edge of the cylinder must, of course, be plane, and the bevel is probably an advantage.

Incubation. The plates are placed on a slab of wood or asbestos and transferred to the 37° incubator for 16–24 hr. If the plates are placed directly on the shelf of the incubator, fluid may condense on the lid of the dish, touch the cylinders and cause their non-sterile contents to run down on to the agar. Incubation is usually allowed to proceed overnight (16 hr.) but the zones of inhibition are quite distinct after less than 10 hr. and, if left longer, increase in size only very slightly.

A modification of this procedure which is sometimes used, especially with solutions of low activity, is to place the prepared plate in the refrigerator for 2–8 hr. before incubation. This allows the penicillin partly to diffuse out before the bacteria begin to multiply rapidly, and the zones are somewhat bigger than would otherwise be the case.

By using plates pre-seeded with spores of a quick growing organism of the *B. subtilis* type, Dr C. G. Pope states (private communication) that results can be read after only 4–5 hr. incubation.

Measurement of zones of inhibition. The diameter of the zones of inhibition (which, in millimetres, we have called the 'assay value') can be measured in several ways. The plates may be turned over and the zone measured directly against a millimetre scale laid on the bottom of the dish. Or the plate

may be placed on a transparent scale illuminated from underneath. A more accurate way is to measure the zones with pointed calipers or dividers, the readings being taken off a paper scale which can be burnt, or a metal scale which can be flamed.

Arranging standard and unknown solutions on the plates. In preparing the plates a number of factors such as batch of medium, length of autoclaving, drying time of plates, number of times the incubator is opened during drying, density of bacterial suspension used for seeding, etc., are not all easy to control in practice. It has been found that the shape of the curve relating assay value to concentration of penicillin, as well as the absolute assay value for any given sample, may vary from day to day, probably depending on these variables. Preliminary investigations showed that a quantitative study of the effects involved would be a lengthy and difficult task; the method is still therefore largely empirical. It is, however, a simple matter to determine the shape of the curve afresh each day by setting up with the unknowns three (or four) solutions containing 2 units, 1 unit, and $\frac{1}{2}$ (and sometimes $\frac{1}{4}$ unit)/ml. respectively. The assay values obtained are plotted against the number of units/ml., and a smooth curve drawn through the points. Then, by reference to the curve, the number of units/ml. corresponding to the assay values of the unknowns can be read off. Fig. 1 shows a typical curve, and Table 1 gives the actual values from which the standard curves were constructed in a series of consecutive tests. The day-to-day variation in this particular series is not great, but over longer periods much larger drifts and occasional abrupt breaks may occur (e.g. on changing to a new batch of medium). It is for this reason that it is considered necessary to construct a standard curve each day.

A detail of considerable importance is the way in which the cylinders are arranged on the plate. If the same solution is placed in six or eight cylinders arranged symmetrically near the periphery of a plate, the resulting zones of inhibition will usually be of identical size. It sometimes happens, however, that the zones nearer the edge to which the plate has been drained during the seeding process are smaller than the average, while those on the opposite side are larger than average, the deviation occasionally reaching as much as ± 2 mm. The opposite of this effect has, on rare occasions, been observed. The causes of this occasional asymmetry

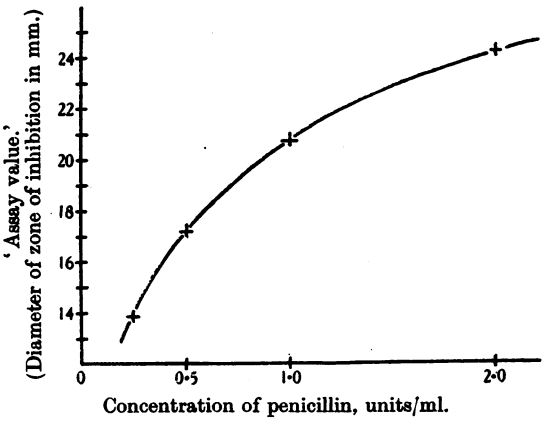

Fig. 1.

are complex and not understood. In the early days replicate assays were usually set up in an identical fashion on successive plates (i.e. each sample would always be in the same position relative to the side to which the plate was drained). Good agreement between replicates was obtained, but it will be realized that if the above-mentioned effect came into play, the mean value for each sample would have a bias depending on the position of that sample on the plates. Errors from this cause can be eliminated or minimized by suitably arranging the replicates on the successive plates. Thus in a quadruplicate assay the samples are arranged in the same order on all four plates, but on each successive plate the series is begun 90° farther round relative to the side to which the seeding fluid was

Table 1. *Assay values produced by standard solutions containing known concentrations of penicillin in nine consecutive tests*

No. of test	Diameter of zone of inhibition in mm. ('assay values') for penicillin solutions containing: 2 units/ml. — Individual data	2 units/ml. — Mean	1 unit/ml. — Individual data	1 unit/ml. — Mean	0·5 unit/ml. — Individual data	0·5 unit/ml. — Mean
1	22·8, 23·6, 22·3, 21·0	22·4	20·2, 21·2, 18·4, 19·1	19·7	16·1, 17·7, 15·1, 13·4	15·6
2	23·8, 23·3, 23·6, 23·5	23·65	18·8, 19·8, 19·6, —	19·4	15·1, 17·8, 15·3, 17·0	16·3
3	22·3, 22·2, 23·3, 23·0	22·7	19·1, 19·2, 19·1, 20·3	19·4	15·1, 15·7, 17·5, 15·9	16·05
4	22·2, 23·2, 22·7, 21·6	22·4	21·0, 17·8, 19·8, 19·7	19·6	17·4, 15·9, 16·6, 15·6	16·4
5	22·9, 23·2, 23·2, 22·8	23·0	20·0, 19·8, 20·2, 17·8	19·45	16·9, 16·2, 17·9, 14·0	16·25
6	23·0, 23·4, 23·7, 24·6	23·7	20·2, 20·6, 20·2, 21·3	20·6	16·4, 17·1, 15·3, 16·9	16·4
7	22·1, 23·3, 22·2, 22·1	22·4	18·8, 18·6, 18·8, 19·4	18·9	17·8, 15·4, 16·1, 17·1	16·6
8	24·3, 24·1, 23 · 22·0	23·6	21·0, 20·2, 19·3, 20·6	20·3	14·8, 16·5, 16·6, 15·6	15·9
9	23·1, 24·7, 23·1, 24·0	23·7	19·5, 20·4, 19·2, 20·1	19·8	14·2, 15·1, 15·3, 15·2	14·95

drained. If this procedure is adopted there will often be a wider scatter in the individual values for one sample, but their *average* values for successive days or runs will agree better.

THE PENICILLIN STANDARD

It is now generally accepted that, for this and most other methods of assay of penicillin, accurate results can be ensured only by comparing the unknowns against a standard of known potency. The first requisite of such a standard is stability, and it has been suggested that mercuric chloride, proflavine or some other stable and easily characterized inhibitor should be used. The arguments against such a course are: (*a*) different strains of test organism will probably respond differently to the proposed standard and to penicillin, which, after all, behaves like no known antiseptic; (*b*) even if a standard test organism is used there is no reason to suppose that if its sensitivity to penicillin changed—and such changes are known to occur spontaneously—its sensitivity to the standard would change in a similar way (Rammelkamp & Maxon, 1942; McKee & Houck, 1943); (*c*) the response to two quite different chemical substances will probably not be identical when slight uncontrolled variations occur in the conditions of the test (see above: *Arranging solutions on the plates*).

For these reasons penicillin itself was chosen as the standard. In 1940 a purely arbitrary unit for internal use in this laboratory was adopted as that amount of penicillin contained in 1 ml. of a certain phosphate buffer solution containing ether. In 1941 a dry sodium salt (containing 42 units/mg.) was standardized against this, and later another primary standard—a barium salt containing 4·4 units/mg.—was prepared. Though substandards in this laboratory and elsewhere have undoubtedly deteriorated, by good fortune the above two primary standards have shown no detectable loss of potency over many months, even when portions were transported to hot climates. The 'Oxford unit' has been adopted by a number of other workers.

Attention may be drawn here to the statement by Florey & Jennings (1942) that 'For those using the dilution method it may be stated that the "Oxford unit" is that amount of penicillin which when dissolved in 50 ml. of meat extract broth just inhibits completely the growth of the test strain of *Staphylococcus aureus*.' It should be pointed out that this method of standardization, though it may give consistent results in the hands of any one group of workers—but see Foster & Woodruff (1943*a*)—is subject to many variables (quality of broth, duration of incubation, etc.) and cannot be taken as necessarily accurate under the conditions prevailing elsewhere.

REPRODUCIBILITY: LIMITS OF ACCURACY

A quantitative idea of the reproducibility of the method is afforded by a series of 27 assays of a given preparation against the same standard. Each single potency value was derived from the mean of four zone measurements, the values being 75, 75, 66, 77, 88·5, 63, 68, 72, 77·5, 77, 77, 85·5, 64·5, 72·5, 76, 66·5, 69, 71·5, 81·5, 65, 71·5, 69, 73·5, 79, 63·5, 81, 73. The coefficient of variation of this series (*Series A*) is 9·0%. Two further series with ten assays in each were run in an exactly similar way with the following results: *Series B*: 56, 56, 62, 61, 51·5, 56·5, 57, 55, 56, 60; coefficient of variation, 5·6%. *Series C*: 70·5, 62, 72, 68·5, 67, 72, 65, 71·5, 65, 68·5; coefficient of variation, 5·0%. In a more detailed statistical analysis of the first series, Dr O. L. Davies, of Imperial Chemical Industries Ltd., found that the error calculated from the variation within single tests was substantially the same as that calculated from variation between tests.

The scatter within tests and between successive tests is illustrated by Table 1, which gives the zone measurements actually recorded for the standard solutions set up in a consecutive series of assays. The unknown solutions would show a similar degree of scatter.

If 9% is taken as the coefficient of variation which may be expected, then for $P = 0{\cdot}01$ the limits of accuracy for a single assay in quadruplicate (i.e. a value derived from measurement of four zones) will be $\pm 23{\cdot}2\%$. For an assay in triplicate the limits will rise to $\pm 26{\cdot}9\%$ and for an assay derived from two zone measurements only they will be $\pm 36{\cdot}7\%$. For $P = 0{\cdot}05$, the limits will be $\pm 19{\cdot}5\%$, $\pm 22{\cdot}7\%$ and $\pm 27{\cdot}7\%$ respectively. In series B and C the degree of reproducibility was considerably greater than this, though the reason is not known.

POSSIBLE MODIFICATIONS AND APPLICATIONS OF THE METHOD

Substitutes for cylinders. Foster & Woodruff (1943*a*) mention that Dowdy, Vincent & Vincent have used, instead of cylinders, circular disks of filter paper which are merely placed on the surface of a seeded plate and impregnated with the solution to be tested. This same method (using disks of thick filter fabric) was proposed in 1940 by Dr C. G. Pope (private communication). In the hole or cup method, which has been used by Fleming (1942) for many years, the solutions to be tested are simply placed in holes punched in a bulk-seeded plate by means of a cork borer and sealed at the bottom with two drops of melted agar. Wilkins & Harris (1943) have used a similar method for studying antibiotics of fungal origin.

Little is known of the upper limits of accuracy of these methods, but a possible disadvantage of all of them is the absence of a barrier to prevent any bacteria contaminating the test samples from spreading over the surface of the plate.

Application to substances other than penicillin. Great caution must be exercised before placing reliance on results obtained by this method when applied to other antibiotics. If the inhibitor is not diffusible, the method is obviously of no use. The composition of the agar may need special attention (e.g. the presence of peptones would presumably interfere with sulphonamides; penicillin B or notatin gives large zones of inhibition in the presence of glucose, but none in its absence). With some substances (e.g. helvolic acid (Chain, Florey, Jennings & Williams, 1943)), the curve relating diameter of inhibition zone to concentration of inhibitor is too flat to be of much use for quantitative work. Results with sparingly soluble inhibitors may need special interpretation. Change in pH of the medium during growth of the bacteria may influence the assay value in a variety of ways. If the potency of the inhibitor depends on the number of bacteria being inhibited, special procedures for ensuring even and reproducible seeding of the plates may be required. Other potential limitations of the method can be imagined, but the method has been successfully applied to the quantitative assay of gigantic acid (Philpot, 1943) and of substances of plant and fungal origin quite different in character from penicillin. The method has been of use in the semi-quantitative investigation of several other substances.

SUMMARY

1. Full practical details are given of a method of assay of penicillin which is applicable also to certain other antibiotics.

2. The method compares favourably as regards speed and accuracy with most other methods and has been in routine use for nearly 4 years.

3. Less than 1 ml. of solution (which need not be sterile) is required for an assay and the presence of ether or chloroform does not interfere.

4. Quantitative information on limits of accuracy is given.

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Technical Notes on the Partition Chromatography of Acetamino-acids with Silica Gel

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We record here some technical aspects of the experience which we have gained in the use of our partition chromatographic method (Gordon, Martin & Synge, 1943*a*) for the quantitative analysis of amino-acid mixtures.

PREPARATION OF SILICA GEL

The characteristics of different batches of silica gel have been found to vary so much as a result of differences in starting material and minor variations in preparative procedure that we have introduced a standard test to which